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(54) Title: NOVEL, PROTRACTED INSULIN ANALOGUES

(57) Abstract

Human insulin analogues wherein at least one of the amino acid residues in position B1-B6 has been replaced by a Lys or Arg have a prolonged insulin action. Asn in position A21 may be replaced by another amino acid residue to increase the stability of the insulin analogue in acid solution. Furthermore B30 may be blocked by means of an amido or ester group.

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NOVEL, PROTRACTED INSULIN ANALOGUES

Field of invention

The present invention relates to novel insulin analogues with a prolonged insulin action, to a process for the preparation of such insulin analogues and to injectable solutions containing the novel insulin analogues.

Background of the invention

Insulin analogues with a protracted insulin action have previously been described in EP 0194864A and EP 0254516A.

In EP 0194864A protracted human insulin analogues wherein the C-terminal carboxyl group of the B-chain is blocked with an amido or ester residue and the amino acid residue in position A4, A17, B13 and B21 may be substituted by Gln are described. EP 0254516A describes human insulin of the same type as in EP 0194486A but further being modified in the A21 position.

Some of the above insulin analogues may, however, show a too low biological potency or the level of prolongation may be too low for specific purposes.

It is the purpose of the present invention to develop novel insulin analogues with prolonged insulin action not suffering from the above-mentioned drawbacks.

Summary of the invention

It has according to the present invention surprisingly been found that introduction of a positive charge in the N-terminal end of the B-chain gives rise to insulin analogues with a highly prolonged insulin action as compared to human insulin and also a high in vivo potency.

In its broadest aspect the present invention is thus related to novel analogues of human insulin wherein at least one of the amino acid residues from B1 to B6 has been

replaced by a basic amino acid residue, i.e. a lysine or arginine residue (Lys or Arg).

For the purpose of improving the stability of the novel insulin analogues asparagine (Asn) in position A21 may furthermore be substituted with another amino acid residue.

Also a further positive charge may be introduced by blocking the C-terminal carboxyl group in position B30 preferably by means of an amido or ester group.

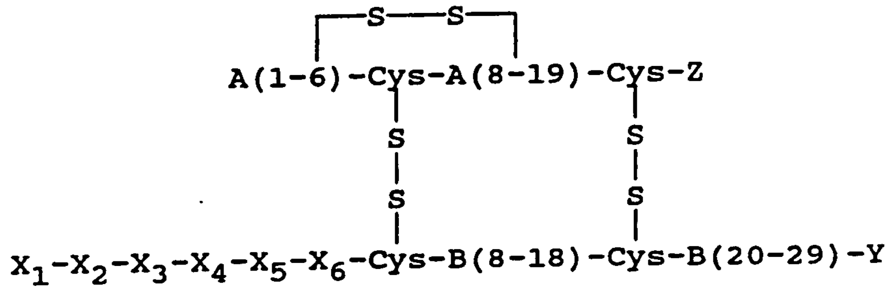
If the present insulin analogues are prepared by the socalled transpeptidition method (for details see later) it might furthermore be an advantage that the amino group linked to the C-terminal end of the Lys or Arg residue substituent is a proline residue.

The invention is also related to a method for the preparation of the novel insulin analogues by which a biosynthetic precursor of the insulin analogue is converted into the insulin analogues by enzymatic and chemical conversion and to insulin solutions containing the novel insulin analogues.

By "insulin analogues" as used herein is meant a compound having a molecular structure similar to that of insulin including the disulphide bridges between Cys^{A7} and Cys^{B7}, and between Cys^{A20} and Cys^{B19} and an internal disulphide bridge between Cys^{A6} and Cys^{A11} and with insulin activity.

<u>Detailed description of the invention</u>

The present insulin analogues may be represented by the following formula I



(I)

wherein Z is Asn or another naturally occuring amino acid residue, X_1 is Phe, Lys or Arg, X_2 is Val, Lys or Arg, X_3 is Asn, Lys, Arg or Pro, X_4 is Gln, Lys, Arg or Pro, X_5 is His, Lys, Arg or Pro, X_6 is Lys, Arg, Leu or Pro and Y is a threonine residue wherein the carboxyl group may be blocked by an ester or amido group, with the proviso that at least one of X_1, X_2, X_3, X_4, X_5 and X_6 is Lys or Arg.

Compared with human insulin, the change in charge is obtained by substituting one or more of the amino acid residues in position B1 to B6 with an arginine or lysine residue. In addition, the C-terminal carboxyl group of the B-chain may be blocked by an ester group or amide group.

If Z is not asparagine it may be a neutral amino acid, for example valine, glutamine, isoleucine, leucine, phenylalanine, tyrosine, methionine or preferably glycine, serine, threonine or alanine. Z may also be an acidic amino acid, viz. glutamic acid or aspartic acid, or a basic amino acid, viz. lysine, arginine or histidine. Z is preferably glycine, alanine or serine.

Examples of blocking groups of the C-terminal carboxyl group in the B30 amino acid residue (threonine) are ester moities such as lower alkoxy with preferably not more than 8 carbon atoms, preferably less than 5 carbon atoms. Preferred alkoxy groups are methoxy, ethoxy and tertiary butoxy.

The blocking group may also be an amido group with the formula $-NR^1R^2$ wherein R^1 and R^2 are the same or

different and each represents hydrogen or alkyl with preferably up to 8 carbon atoms. R^1 and R^2 are preferably each hydrogen.

Since compounds of formula I can be applied in the clinic as solutions having a prolonged action, a decline in immunogenicity as compared to the commonly used suspensions of porcine or human insulins may occur.

The degree of prolongation can be enhanced and controlled by the addition of zinc ions.

Parameters that may control the degree of prolongation of the insulin effect are the concentration of zinc and the choice of the compound of formula I. The range for preferred zinc content extends from 0 to about 2 mg/ml, preferably from 0 to 200 μg/ml zinc and more preferably from about 20 to 200 μg/ml in a preparation containing about 240 nmole of a compound of formula I per ml. Using other concentrations of the compound of formula I, the content of zinc is to be adjusted correspondingly.

The prolonged action of solutions of compounds of formula I in the presence of zinc ions is ascribed to the low solubility of such compounds at neutral pH.

The pH of the injectable solution of this invention should preferably be below the physiological pH, the upper limit being the pH where precipitation occurs. At the physiological pH value, compounds of formula I of this invention have a low solubility. Stable solutions containing about 240 nmole/ml of compounds of formula I per ml have been obtained at pH about 5.5. The upper limit depends upon the constituents of the solution, i.e. isotonikum, preservative and zinc concentration, and upon the choice of compound of formula I. There is no lower pH limit of the solutions and the chemical stability of the compounds of formula I where Z is different from asparagine, is high, even at pH 3. The preferred pH range for the injectable solutions of this invention is from about 2.5 to 8.5, more preferred from about

4.5 to 8. Especially preferred are pH ranges about 2.5 to 5.5, most prefered about 3 to 4.5.

A furter aspect of this invention is that it provides improved flexibility for the patients. With two aqueous solutions, one containing a compound of formula I and the other containing a zinc salt, the patient can obtain a desired degree of prolonged action and a desired profile by mixing the two solutions appropriately. Thus, the patient has, using two stock solutions, the possibility of choosing one action and profile for the morning injection and another action and profile for the evening injection. Preferably, the zinc solution of this invention contains between about 2 μg and 20 mg zinc per ml. Alternatively, both of the stock solutions may contain zinc, either in the same or different concentrations, and/or both the stock solutions may contain a compound of formula I, either the same or different compounds.

Perferably, the injectable solutions of this invention have a strength of between about 60 and 6000 nmole of the compound of formula I per ml.

Examples of novel insulin analogues according to the present invention are

$Arg^{B5},Ser^{A21},Thr^{B30}-NH_2$ human insulin
$Arg^{B5},Pro^{B6},Ser^{A21},Thr^{B30}-NH_2$ human insulin
$Arg^{B5},Gly^{A21},Thr^{B30}-NH_2$ human insulin
$Arg^{B5},Pro^{B6},Gly^{A21},Thr^{B30}-NH_2$ human insulin
$Arg^{B2},Ser^{A21},Thr^{B30}-NH_2$ human insulin
$Arg^{B2},Pro^{B3},Ser^{A21},Thr^{B30}-NH_2$ human insulin
$Arg^{B2},Gly^{A21},Thr^{B30}-NH_2$ human insulin
$Arg^{B2},Pro^{B3},Gly^{A21},Thr^{B30}-NH_2$ human insulin
$Arg^{B2},Arg^{B3},Ser^{A21},Thr^{B30}-NH_2$ human insulin
$Arg^{B2},Arg^{B3},Ser^{A21}$ human insulin
$Arg^{B4},Pro^{B5},Ser^{A21},Thr^{B30}-NH_2$ human insulin
$Arg^{B4},Arg^{B5},Pro^{B6},Gly^{A21},Thr^{B30}$ human insulin

$Arg^{B3}, Gly^{A21}, Thr^{B30}-NH_2$ human insulin
$Arg^{B3}, Ser^{A21}, Thr^{B30}-NH_2$ human insulin
$Arg^{B4}, Gly^{A21}, Thr^{B30}-NH_2$ human insulin
$Arg^{B4}, Ser^{A21}, Thr^{B30}-NH_2$ human insulin
$Arg^{B1}, Pro^{B2}, Gly^{A21}, Thr^{B30}-NH_2$ human insulin.

The novel insulin analogues according to the present invention may be prepared by altering the proinsulin gene through replacement of codon(s) at the appropriate site in the native human proinsulin gene by codon(s) encoding the desired amino acid residue substitute(s) or by synthesizing the whole DNA-sequence encoding the desired insulin analogue. The gene encoding the desired insulin analogue is then inserted into a suitable expression vector which when transferred to a suitable host organism, e.g. E. coli, Bacillus or yeast, generates the desired product. The expressed product is then isolated from the cells or the culture broth depending on whether the expressed product is secreted from the cells or not.

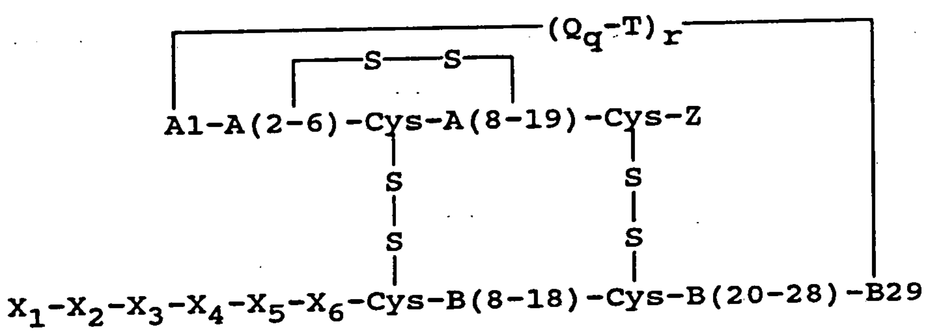
The novel insulin analogues may also be prepared by chemical synthesis by methods analogue to the method described by Märki et al. (Hoppe-Seyler's Z. Physiol.Chem., 360 (1979), 1619-1632). They may also be formed from separately in vitro prepared A- and B-chains containing the appropriate amino acid residue substitutions, whereupon the modified A- and B-chains are linked together by establishing disulphide bridges according to known methods (e.g. Chance et al., In: Rick DH, Gross E (eds) Peptides: Synthesis - Structure - Function. Proceedings of the seventh American peptide symposium, Illinois, pp. 721-728).

The insulin analogues may furthermore be prepared by a method analogue to the method described in EP 0163529A, the disclosure of which is incorporated by reference hereinto. By such a method an insulin precursor of human insulin wherein Lys^{B29} is connected to Gly^{A1} by means of either a peptide

bond or a peptide chain of varying length with correctly positioned disulphide bridges is expressed and secreted by yeast and then converted into human insulin by the so-called transpeptidation reaction.

The transpeptidation reaction is described in US patent specification No. 4,343,898 (the disclosure of which is incorporated by reference hereinto). In this reaction the peptide bond or peptide chain connecting Lys^{B29} and Gly^{A1} is exised and a threonine ester or threonine amide group is coupled to the C-terminal end of Lys^{B29}.

The novel insulin analogues may thus be prepared by a method wherein a biosynthetic insulin precursor with the following formula II



(II)

wherein Q is a peptide chain with q amino acid residues, q is an interger from 0 to 33, T is Lys or Arg, r is 0 or 1 and X_1, X_2, X_3, X_4, X_5, X_6 and Z are defined as above, is reacted with a compound of the formula III

HY (III)

wherein Y is a protected threonine amino acid wherein the carboxyl group is protected with an ester or amido group, using trypsin or trypsin like enzymes as a catalyst in a mixture of water and organic solvent. The ester or amido protecting group may then be cleaved off by acid or basic hydrolysis.

Preferred compounds of formula III are Thr-NH_2, Lys(Boc)-NH_2, Thr(Bu^t)-OBu^t and Thr-OBu^t.

Insulin preparations of this invention are prepared by dissolving a compound of formula I in an aqueous medium at slightly acidic conditions, for example, in a concentration of 240 or 600 nmole/ml. The aqueous medium is made isotonic, for example, with sodium chloride or glycerol. Furthermore, the aqueous medium may contain zinc ions in a concentraion of up to about 30 μg of Zn^{++} per nmol of compound of formula I, buffers such as acetate, citrate and histidine and preservatives such as m-cresol, nipagin or phenol. The pH value of the final insulin preparation depends upon the number of charges that have been changed in the compound of formula I, the concentration of zinc ions, the concentration of the compound of formula I and the compound of formula I selected. The pH value is adjusted to a value convenient for administration such as about 2.5 - 5.5, preventing precipitation. The insulin preparation is made sterile by sterile filtration.

The insulin preparations of this invention can be used similarly to the use of the known insulin preparations.

Terminology

The abbreviations used for the amino acids are those stated in J.Biol.Chem. 243 (1968), 3558. The amino acids are in the L configuration. Unless otherwise indicated, the species of insulins stated herein is human.

As used in the following text B(1-29) means a shortened B-chain of human insulin from Phe^{B1} to Lys^{B29} and A(1-21) means the A-chain of human insulin.

The substitution(s) made in the huamn insulin molecule according to the practice of the invention is (are) indicated with a prefix referenced to human insulin. As an example Arg^{B2} human insulin means a human insulin analogue wherein Arg has been substituted for Val in position 2 in the

B-chain. Arg^{B2},B(1-29)-Ala-Ala-Lys-A(1-21) human insulin means a precursor for the forementioned insulin analogue wherein Arg has been substituted for Val in position 2 in the shortened B-chain and wherein the B(1-29) chain and the A(1-21) chain are connected by the peptide sequence Ala-Ala-Lys.

Unless otherwise stated it is to be understood that the B(1-29) chain and the A(1-21) chain are connected by disulphide bridges between Cys^{A7} and Cys^{B7} and between Cys^{A20} and Cys^{B19}, respectively, and that the A-chain contains an internal disulphide bridge between Cys^{A6} and Cys^{A1}, as in human insulin.

Experimental part

Example 1

Arg^{B2},Pro^{B3},Ser^{A21},Thr^{B30}-NH_2 human insulin,
Arg^{B5},Ser^{A21},Thr^{B30}-NH_2 human insulin,
Arg^{B4},Gly^{A21},Thr^{B30}-NH_2 human insulin and
Arg^{B2},Pro^{B3},Gly^{A21},Thr^{B30} human insulin
Arg^{B3},Gly^{A21},Thr^{B30}-NH_2 human insulin

The above compounds were synthesized from the corresponding the following precursors:
Arg^{B2},Pro^{B3},Ser^{A21},B(1-29)-Ala-Ala-Lys-A(1-21) human insulin,
Arg^{B5},Ser^{A21},B(1-29)-Ala-Ala-Lys-A(1-21) human insulin,
Arg^{B4},Gly^{A21},B(1-29)-Ala-Ala-Lys-A(1-21) human insulin,
Arg^{B2},Pro^{B3},Gly^{A21},B(1-29)-Ala-Ala-Lys-A(1-21) human insulin,
and
Arg^{B3},Glu^{A21},B(1-29)-Ala-Ala-Lys-A(1-21) human insulin
by tryptic transpeptidation in organic/aqueous solution in the presence of Thr-NH_2 as described in EP 0194864A, Examples 4 and 6. Yields, charges relative to human insulin, rates of migration relative to insulin in DISC PAGE electrophoresis at pH 8.9 and deviations in amino acid compositions from human insulin appear from Table I.

The insulin precursors were produced by a method analogous to the method described in EP 0163529A.

The insulin precursors were recovered from the fermentation broths by adsorption of LiChroprep™ RP-18 as described in Example 7 of EP 0163529A. The precursors were eluted from the column with 0.2 M KCl, 0.001 M HCl in 33% (v/v) ethanol. The insulin precursors were crystallized from the pool by successive additions of water (1 volume per volume of pool), solid trisodium citrate to obtain a molarity of 0.05 M and finally zinc acetate to obtain a molarity of 0.006 M. The pH value was adjusted to 6.8 and the mixture was left overnight at 4°C. The crystals were isolated by centrifugation, washed with water and dried in vacuo.

Protected amino acids and protected peptides for enzymatic semisynthesis were either prepared by standard methods or purchased (custom synthesis) from either Nova Biochem or Bachem, both Switzerland.

Table I

Substitution in human insulin	Yield %	Charge relative to human insulin at pH 7	Rate of migration at pH 8.9, % relative to human insulin	Deviations in amino acid compositions from human insulin after acid hydrolysis residues/mole
Arg^{B2}, Pro^{B3}, Ser^{A21}, Thr^{B30}-NH_2	27	+2	55	+1 Arg, +1 Pro, +1 Ser, -2 Asp, -1 Val
Arg^{B5}, Ser^{A21}, Thr^{B30}-NH_2	54	+2	55	+1 Arg, +1 Ser, -1 Asp, -1 His
Arg^{B4}, Gly^{A21}, Thr^{B30}, NH_2	48	+2	55	+1 Arg, +1 Gly, -1 Glu, -1 Asp
Arg^{B2}, Pro^{B3}, Gly^{A21}, Thr^{B30}-NH_2	57	+2	55	+1 Arg, +1 Pro, +1 Gly, -1 Val, -2 Asp

Sterile injectable solutions of the above compounds for testing of the degree of prolonged action were made using 1.6% (w/v) glycerol as the isotonicum, and 0.26% (w/v) phenol as the preservative. The concentration of zinc ions was 8, 80 or 160 μg/ml. The pH values of the solutions were adjusted sufficiently off the isoelectric point of the analogues to keep the solutions clear upon storage at 4°C. The solutions contained 240 nmole/ml of the tested compounds. The concentration of 240 nmole/ml was verified by HPLC.

Injectable solutions containing 240 nmole/ml of the compounds stated in Table II and having the pH of 3 and content of zinc stated therein were then made.

The prolongation of the hypoglycemic effect was tested according to British Pharmacopoeia <u>1980</u>, A 142, in fasted rabbits. Each test solution was administered subcutaneously in a dosis of 14.3 nmole per rabbit in 12 animals weighing 3 - 4 kg, and the course of the hypoglycemia was followed for 6 hours. For comparison the fast acting preparation, Actrapid^{TM} human insulin was included in the tests. The results of the tests are shown in Table II giving the percentage of glucose after 1, 2, 4 and 6 hours (h). Results from determination of biological potencies Mouse Blood Glucose Assay (MBG) and Free Fat Cell Test (FFC) are listed in Table III.

Table II

Compound	Zn^{++}	Glucose in percent of initial			
Substitutions in human insulin	µg/ml	1h	2h	4h	6h
Arg^{B5},Ser^{A21},Thr^{B30}-NH_2	8	59	64	96	94
Arg^{B5},Ser^{A21},Thr^{B30}-NH_2	80	85	92	97	95
Arg^{B5},Ser^{A21},Thr^{B30}-NH_2	160	76	89	96	94
Arg^{B2},Pro^{B3},Ser^{A21},Thr^{B30}-NH_2	8	58	67	73	72
Arg^{B2},Pro^{B3},Ser^{A21},Thr^{B30}-NH_2	80	73	66	64	61
Actrapid™ human insulin	7	53	53	92	97

Table III

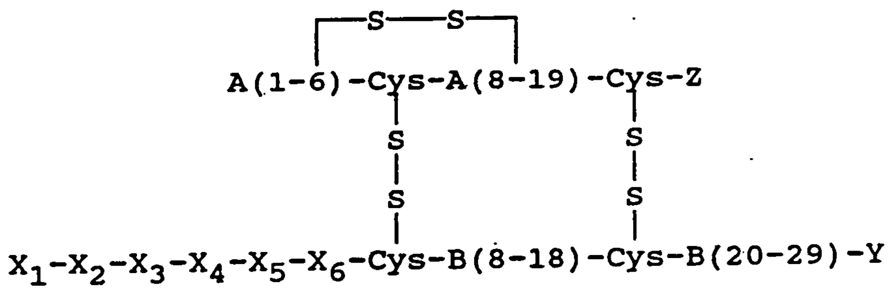
Biological potency relative to human insulin.

(British Pharmacopoeia 1980, A141 - A142).

	MBGA		FFC	
Substitutions in human insulin	Potency relative to human insulin, %	Confidence limits, % (P = 0.05)	Potency relative to human insulin, %	Confidence limits, % (p = 0.05)
$Arg^{B5}, Ser^{A21}, Thr^{B30}-NH_2$	76	86-68	56	54-58
$Arg^{B2}, Pro^{B3}, Ser^{A21}, Thr^{B30}-NH_2$	56	49-64	50	47-52
$Arg^{B2}, Pro^{B3}, Gly^{A21}, Thr^{B30}-NH_2$			59	57-61
$Arg^{B4}, Gly^{A21}, Thr^{B30}-NH_2$	66	60-72	40	38-41

CLAIMS

1. Human insulin analogues having the following formula I



(I)

wherein Z is Asn or another naturally occuring amino acid residue, X_1 is Phe, Lys or Arg, X_2 is Val, Pro, Lys or Arg, X_3 is Asn, Lys, Arg or Pro, X_4 is Gln, Lys, Arg or Pro, X_5 is His, Lys, Arg or Pro, X_6 is Lys, Arg, Leu or Pro and Y is a threonine residue wherein the carboxyl group may be blocked by an ester or amido group, with the proviso that at least one of X_1, X_2, X_3, X_4, X_5 and X_6 is Lys or Arg.

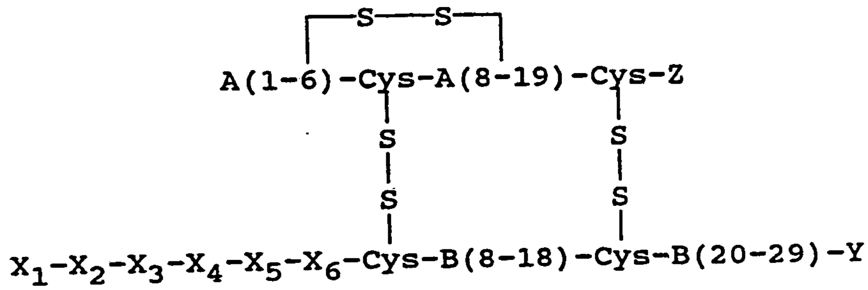
2. Human insulin analogues according to claim 1, wherein X_1 is Phe.

3. Human insulin analogues according to claim 1 or 2 wherein Z is Gly, Ala or Ser.

4. Human insulin analogues according to claim 1 wherein X_1 is Phe, X_2 is Arg or Lys, X_3 is Pro, X_4 is Gln, X_5 is His, X_6 is Leu, Z is Gly, Ala or Ser and Y is Thr-NH_2 or Thr.

5. Human insulin analogues according to claim 1 wherein X_1 is Phe, X_2 is Val, X_3 is Asn, X_4 is Gln, X_5 is His, X_6 is Arg or Lys, Z is Gly, Ala or Ser and Y is Thr-NH_2 or Thr.

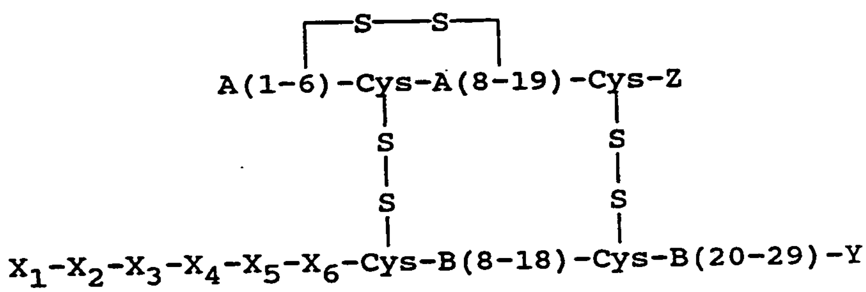
6. Injectable solutions with prolonged insulin action containing a human insulin analogue with the general formula I



(I)

wherein Z is Asn or another naturally occuring amino acid, X_1 is Phe, Lys or Arg, X_2 is Val, Lys or Arg, X_3 is Asn, Lys, Arg or Pro, X_4 is Gln, Lys, Arg or Pro, X_5 is His, Lys, Arg or Pro, X_6 is Lys, Arg, Leu or Pro and Y is a threonine residue wherein the carboxyl group may be blocked by an ester or amido group, with the proviso that at least one of X_1, X_2, X_3, X_4, X_5 and X_6 is Lys or Arg, together with conventional auxiliary agents, such as buffers, preservatives and agents making the solution isotonic.

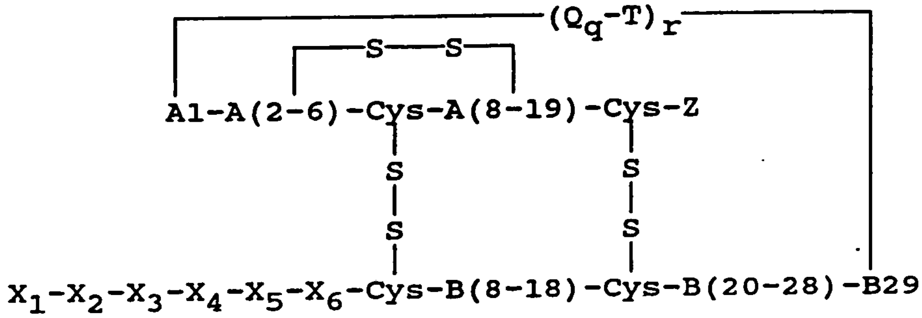
7. A method for making insulin analogues with the following formula I



(I)

wherein Z is Asn or another naturally occuring amino acid residue, X_1 is Phe, Lys or Arg, X_2 is Val, Pro, Lys or Arg, X_3 is Asn, Lys, Arg or Pro, X_4 is Gln, Lys, Arg or Pro, X_5 is His, Lys, Arg or Pro, X_6 is Lys, Arg, Leu or Pro and Y is a threonine residue wherein the carboxyl group may be blocked by an ester or amido group, with the proviso that at least

one of X_1, X_2, X_3, X_4, X_5 and X_6 is Lys or Arg, wherein an insulin percursor with the following formula II



(II)

wherein Q is a peptide chain with q amino acid residues, q is an interger from 0 to 33, T is Lys or Arg, r is 0 or 1 and X_1, X_2, X_3, X_4, X_5, X_6 and Z are defined as above, is reacted with a compound of the formula III

HY (III)

wherein Y is a protected threonine amino acid residue wherein the carboxyl group is protected with an ester or amido group, using trypsin or trypsin like enzymes as a catalyst in a mixture of water and organic solvent, whereupon the protecting group if desired is cleaved off by acid or basic hydrolysis.

INTERNATIONAL SEARCH REPORT

International Application No PCT/DK 91/00167

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 07 K 7/40, A 61 K 37/26

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	A 61 K; C 07 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched[8]

SE,DK,FI,NO classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	Chemical Abstracts, volume 86, no. 7, 14 February 1977, (Columbus, Ohio, US), Kolomeitseva, L. A. et al.: "Natural peptides and their analogs. IX. Synthesis of Gly5-, Arg5-, and Gly6-analogs of insulin (pig) B-chain. ", see page 566, abstract 44002g, & Zh. Obshch. Khim. 1976, 46(5), 1176-1181 --	1-3
X	Chemical Abstracts, volume 88, no. 19, 8 May 1978, (Columbus, Ohio, US), Shvachkin, Yu. P. et al.: "Preparation of (Arg-B5)-, (Gly-B6)-, (Gly-B5)-analogs of bull insulin ", see page 570, abstract 136957s, & Khim. Prir. Soedin. 1977, 5(), 722- 723 --	1,2
A	EP, A2, 0214826 (NOVO INDUSTRI A/S) 18 March 1987, see the whole document --	1-7

* Special categories of cited documents: [10]

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
4th October 1991	1991 -10- 08
International Searching Authority	Signature of Authorized Officer
SWEDISH PATENT OFFICE	Elisabeth Carlborg

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	EP, A2, 0375437 (NOVO-NORDISK A/S) 27 June 1990, see the whole document --	1-7
A	DE, A1, 2536040 (HOECHST AG) 24 February 1977, see page 2, line 10 - line 14 --	1-7
A	WO, A1, 8910937 (NOVO-NORDISK A/S) 16 November 1989, see the whole document --	1-7

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. PCT/DK 91/00167

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the Swedish Patent Office EDP file on 91-08-30
The Swedish Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A2- 0214826	87-03-18	AU-B-	593274	90-02-08
		AU-D-	6206686	87-03-05
		JP-A-	62053999	87-03-09
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		CA-A-	2006578	90-06-23
		WO-A-	90/07522	90-07-12
DE-A1- 2536040	77-02-24	NONE		
WO-A1- 8910937	89-11-16	AU-D-	3692889	89-11-29
		EP-A-	0419504	91-04-03